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Evaluation of Three Strains of Influenza A Virus in Humans and in Owl, Cebus, and Squirrel Monkeys

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The virulence of three cloned influenza A viruses was compared in humans and in three readily available species of nonhuman primates (owl, squirrel, and cebus monkeys) in an attempt to identify a species of monkey that could be used to investigate the genetic basis of attenuation of influenza A viruses for humans. Three influenza A viruses from two subtypes, i.e., the A/Udorn/72 (H3N2), A/Alaska/77 (H3N2), and A/Hong Kong/77 (H1N1) viruses, produced febrile influenzal illness in humans. Squirrel monkeys developed mild upper respiratory tract illness in response to each of the three viruses. Illness was accompanied by a high level of virus shedding; each of nine squirrel monkeys that shed equal to or greater than $10^{5.0}$ 50% tissue culture infective doses of virus became ill, whereas those that shed less remained well. In contrast, the cebus and owl monkeys remained clinically well despite infection with each of the three viruses. Thus, squirrel monkeys appear to be moderately permissive primate hosts in which to investigate the genetic basis of virulence of human influenza A viruses.

Attenuation of new strains of influenza A virus can be accomplished rapidly by the transfer of attenuating genes from an attenuated donor virus to new epidemic or pandemic strains by genetic reassortment (7a). However, evaluation of such recombinants for their usefulness in the immunoprophylaxis of influenza has proceeded slowly since volunteers remain the most reliable indicator of satisfactory attenuation. For this reason, it would be advantageous to identify a simian host in which recombinants could be evaluated in a preliminary manner before tests in humans. Such a simian species would be especially useful for the evaluation of host range mutants since infection of a susceptible nonhuman primate species should most closely approximate that of humans (7a).

During the course of recent studies, we cloned wild-type influenza A virus of two different subtypes, H3N2 and H1N1, and evaluated these viruses in volunteers (8; B. R. Murphy, R. M. Chanock, R. G. Douglas, R. F. Betts, D. H. Waterman, H. P. Holley, Jr., D. L. Hoover, S. Suwanagool, D. R. Nalin, and M. M. Levine, Arch. Virol., in press; Murphy et al., unpublished observations). In the present study, we compared the virulence of three cloned wild-type influenza A viruses in humans and in three readily available species of nonhuman primates (owl, squirrel, and cebus monkeys) in an attempt to identify a species of monkey that could be used to investigate the genetic basis of virulence of influenza A viruses for humans.

MATERIALS AND METHODS

Viruses. The production and clinical evaluation of the influenza A/Udorn/72 (H3N2), A/Alaska/77 (H3N2), and A/Hong Kong/77 (H1N1) wild-type viruses are presented elsewhere (8; Murphy et al., Arch. Virol., in press; Murphy et al., unpublished observation). These viruses were cloned by plaque-to-plaque passage in primary bovine kidney monolayer culture as described previously (8; Murphy et al., Arch. Virol., in press).

Monkeys. Monkeys were selected for study if their serum hemagglutination-inhibiting antibody titer was less than or equal to 1:8 for the virus under study. The 20 owl monkeys (*Aotus trivirgatus*) and 20 squirrel monkeys (*Saimiri scurucus*) employed in this investigation were feral adults which had been quarantined and conditioned in the laboratory for at least 6 months before inoculation. The 20 cebus monkeys (*Cebus apella* and *Cebus albifrons*) were selected from laboratory-raised juveniles (6 months to 1 year old). All animals were healthy at the start of the experiment. Compatible pairs of monkeys were housed in airflow-controlled isolation cages (6). Routine feeding and care were performed as previously described (5).

Experimental procedure. Each virus was inoculated into six monkeys of each species. Two animals of each species were inoculated with normal allantoic fluid to serve as controls. The monkeys were lightly anesthetized with ketamine hydrochloride administered intramuscularly before experimental inoculation. With a 23-gauge needle, 0.5 ml of virus or control

fluid was inoculated transtracheally through surgically prepared skin immediately below the cricoid cartilage. None of the control monkeys became infected or ill. The quantity of virus administered is shown in Table 1. Animals were monitored twice daily for signs of illness. Rectal temperatures were monitored daily for the first 10 days and also on days 14 and 21. A combined nasal and throat swab was obtained daily for the first 10 days and on days 14 and 21 post-inoculation and placed in veal infusion broth containing 66 μg of gentamicin per ml, 10 μg of amphotericin per ml, and 0.5% gelatin. Swab fluid was inoculated fresh, in 0.1-ml quantities, onto four wells of a 24-well Co-star tissue culture plate containing Madin-Darby canine kidney (MDCK) cells (Flow Laboratories, Inc., Rockville, Md.) supplemented with 1 μg of tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (Worthington Biochemicals Corp., Freehold, N.J.) per ml and tested for hemadsorption after 5 days (Murphy et al., Arch. Virol., in press). Frozen samples of the nasal and throat swab fluid were titrated on MDCK 24-well plates, using four wells per decimal dilution. Titers were expressed as 50% tissue culture infective doses per milliliter of swab specimens. Anterior-posterior and lateral chest radiographs were taken before virus administration and on days 2, 6, and 14 thereafter. Serum was collected from the animal before inoculation and at 7, 14, 21, and 28 days after virus administration.

Hemagglutination inhibition test. The hemagglutination inhibition test was performed in microtiter plates; 4 antigen units of each virus, the A/Udorn/72 (H3N2), A/Alaska/77 (H3N2), or A/Hong Kong/77 (H1N1), was used.

Criteria for designation of illness. The following categories of illness were used to define a monkey's clinical response. Upper respiratory tract illness was diagnosed when rhinorrhea or sneezing or both were observed on at least 2 consecutive days. Systemic illness was defined as one or more of the following: radiographic evidence of pneumonia, dyspnea, lethargy, and anorexia. The criteria for the designation of illness in volunteers have been described previously (8).

RESULTS

Clinical, virological, and immunological evaluations of influenza A virus infection in monkeys. The clinical, virological, and immunological evaluations of three influenza A wild-type viruses in four species of primates are shown in Table 1. Each of the three wild-type viruses induced febrile respiratory tract disease in humans at approximately $10^{4.0}$ 50% tissue culture infective doses. Illness did not develop in owl or cebus monkeys during infection with any of the three viruses, despite the administration of a dose 100- to 1,000-fold higher than that given to volunteers. Squirrel monkeys developed illness, but the illness was mild and consisted of afebrile coryza. Radiographic evidence of pneumonia was not observed.

Virus shedding was quantitated by determining the average duration of virus shedding and the maximum amount of virus shed by each monkey or volunteer. For the A/Hong Kong/77 (H1N1) wild-type virus, the quantity of virus shed was greatest for humans and least for cebus monkeys. Cebus monkeys also appeared to be the least permissive for the A/Alaska/77 (H3N2) virus. Squirrel monkeys shed the H3N2 viruses at levels comparable to those of humans; however, shedding of the H1N1 virus was significantly reduced. Each of the nine squirrel monkeys that shed $10^{5.0}$ 50% tissue culture infective doses or more of any one of the three influenza A viruses per ml developed illness, whereas animals that shed less than this quantity remained well. Only two owl monkeys achieved this level of virus shedding, and this was not accompanied by illness. None of the cebus monkeys shed $10^{5.0}$ 50% tissue culture infective doses of virus per ml.

Monkeys mounted a vigorous serum hemagglutination-inhibiting antibody response after infection with influenza A virus, but a quantitative relationship between virus shedding and the magnitude of the hemagglutination-inhibiting response was not apparent.

DISCUSSION

Influenza A viruses have been evaluated for virulence in a variety of nonhuman primate species (1–5, 9, 10). Significant morbidity has been observed in cebus and squirrel monkeys with A/Victoria/75 (H3N2) and A/New Jersey/76 (Hsw1N1) viruses (2, 5), whereas illness has not been a regular feature of infection of rhesus monkeys (1, 7, 9, 10). In the present study, we sought to identify a single species of available nonhuman primate that is suceptible to influenza A viruses belonging to different subtypes. The influenza A viruses chosen for evaluation in the present study were known to be virulent in humans and were cloned suspensions which would allow their subsequent use in studies to investigate the genetic basis of virulence. Of the three species tested, squirrel monkeys developed mild illness confined to the upper respiratory tract in response to three different viruses belonging to the H3N2 or H1N1 subtype. This illness response was accompanied by a high level of virus shedding; each of the nine squirrel monkeys that shed equal to or greater than $10^{5.0}$ 50% tissue culture infective doses of virus per ml developed illness, whereas those that shed less virus remained well. None of the cebus monkeys attained this level of shedding, although two owl monkeys did so without evidence of illness.

Previous studies indicated that squirrel monkeys developed clinically apparent influenzal ill-

TABLE 1. *Virological, immunological, and clinical evaluations of three virulent human influenza A viruses in three nonhuman primate species*[a]

Virus	Species	Dose of virus administered ($TCID_{50}$)	No. studied	No. infected	Virus shedding			Serum HI antibody			No. with indicated illness		
					No. that shed	Avg[b] duration (days ± SE)	Mean[b] peak log_{10} titer	Reciprocal of mean log_2 titer (±SE)		No. with fourfold or greater response	Febrile or systemic	Upper respiratory tract	Total
								Before	After				
A/Hong Kong/77 (H1N1) wild type	Human	$10^{4.2}$	6	6	6	5.8 ± 0.2	6.3 ± 0.3	2.0 ± 0.0	6.2 ± 0.4	6	4	4	5
	Cebus monkey	$10^{6.7}$	6	4	1	0.3 ± 0.3	0.6 ± 0.1	1.0 ± 0.0	4.2 ± 0.9	4	0	0	0
	Owl monkey	$10^{6.7}$	6	6	4	2.8 ± 1.2	3.1 ± 1.0	1.2 ± 0.2	8.3 ± 0.3	6	0	0	0
	Squirrel monkey	$10^{6.7}$	6	5	4	4.2 ± 1.7	3.2 ± 1.2	1.8 ± 0.2	5.3 ± 1.1	5	0	2	2
A/Udorn/72 (H3N2) wild type	Human	$10^{4.0}$	6	5	5	5.9 ± 0.7	3.0 ± 0.8	1.2 ± 0.2	3.3 ± 0.6	4	3	2	5
	Cebus monkey	$10^{7.0}$	6	6	6	7.2 ± 1.3	3.3 ± 0.5	1.2 ± 0.2	6.3 ± 0.8	6	0	0	0
	Owl monkey	$10^{7.0}$	6	5	4	5.8 ± 1.7	2.7 ± 0.6	1.0 ± 0.0	3.8 ± 0.8	4[c]	0	0	0
	Squirrel monkey	$10^{7.0}$	6	6	6	5.8 ± 0.6	5.3 ± 0.2	1.0 ± 0.0	4.8 ± 0.7	5	0	5	5
A/Alaska/77 (H3N2) wild type	Human	$10^{4.2}$	8	8	8	4.8 ± 0.8	4.5 ± 0.6	2.4 ± 0.2	4.1 ± 0.7	6	3	4	4
	Cebus monkey	$10^{6.2}$	6	6	4	3.8 ± 1.7	1.7 ± 0.5	1.0 ± 0.0	7.0 ± 0.5	6	0	0	0
	Owl monkey	$10^{6.2}$	6	6	6	7.2 ± 0.8	4.3 ± 0.4	1.8 ± 0.3	7.8 ± 0.4	6	0	0	0
	Squirrel monkey	$10^{6.2}$	6	5	5	4.4 ± 1.4	3.7 ± 1.2	1.3 ± 0.2	6.5 ± 1.4	4	0	2	2

[a] Abbreviations: $TCID_{50}$, 50% tissue culture infective doses; SE, standard error; HI, hemagglutination inhibiting.
[b] Calculated for infected animals only.
[c] One animal not available for follow-up.

ness when given A/New Jersey/76 (Hsw1N1) or an A/Aichi/68 (H3N2) virus (2, 3). These findings, in combination with the present observations, indicate that squirrel monkeys regularly develop objective upper respiratory illness after infection with influenza A viruses belonging to the H0-H1-Hswine or H3N2 subtype. In contrast, cebus monkeys appear to respond less reproducibly to influenza A virus infection in that only one of three H3N2 viruses and none of two H0-H1-Hswine viruses induced illness. Thus, squirrel monkeys appear to be moderately permissive primate hosts in which to investigate the genetic basis of virulence of human influenza A viruses.

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LITERATURE CITED

1. **Berendt, R. F.** 1974. Simian model for the evaluation of immunity to influenza. Infect. Immun. **9**:101–105.
2. **Berendt, R. F., and W. C. Hall.** 1977. Reaction of squirrel monkeys to intratracheal inoculation with influenza/A/New Jersey/76 (swine) virus. Infect. Immun. **16**:476–479.
3. **Berendt, R. F., G. G. Long, and J. S. Walker.** 1975. Influenza alone and in sequence with pneumonia due to Streptococcus pneumoniae in the squirrel monkey. J. Infect. Dis. **132**:689–693.
4. **Burnet, F.** 1941. Influenza virus "A" infections of cynomolgus monkeys. Aust. J. Exp. Biol. Med. Sci. **19**:281–290.
5. **Grizzard, M. B., W. T. London, D. L. Sly, B. R. Murphy, W. D. James, W. P. Parnell, and R. M. Chanock.** 1978. Experimental production of respiratory tract disease in cebus monkeys after intratracheal or intranasal infection with influenza A/Victoria/3/75 or influenza A/New Jersey/76 virus. Infect. Immun. **21**:201–205.
6. **London, W. T., H. J. Alter, J. Lander, and R. H. Purcell.** 1972. Serial transmission in rhesus monkeys of an agent related to hepatitis-associated antigen. J. Infect. Dis. **125**:382–389.
7. **Marois, P., A. Boudreault, E. DiFranco, and V. Pavilanis.** 1971. Response of ferrets and monkeys to intranasal infection with human, equine and avian influenza viruses. Can. J. Comp. Med. **35**:71–76.

7a. **Murphy, B. R., R. M. Chanock, R. G. Douglas, R. F. Betts, D. H. Waterman, H. P. Holley, Jr., D. L. Hoover, S. Suwanagool, D. R. Nalin, and M. M. Levine.** 1980. Temperature-sensitive mutants of influenza A virus: evaluation of the Alaska/77-*ts*-1A2 temperature-sensitive recombinant virus in seronegative adult volunteers. Arch. Virol. **288**:401–415.

8. **Richman, D. D., B. R. Murphy, R. M. Chanock, J. M. Gwaltney Jr., R. G. Douglas, R. F. Betts, N. R. Blacklow, F. B. Rose, T. A. Parrino, M. M. Levine, and E. S. Caplan.** 1976. Temperature-sensitive mutants of influenza A virus. XII. Safety, antigenicity, transmissibility, and efficacy of influenza A/Udorn/72-*ts*-1[E] recombinant viruses in human adults. J. Infect. Dis. **134**:585–594.
9. **Saslaw, S., and H. Carlisle.** 1965. Aerosol exposure of monkeys to influenza virus. Proc. Soc. Exp. Biol. Med. **119**:838–843.
10. **Saslaw, S., H. Wilson, C. Doan, O. Woolpert, and J. Schwab.** 1946. Reactions of monkeys to experimentally induced influenza virus A infection. J. Exp. Med. **84**: 113–125.